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Preliminary Interpretation of Ruminant Fecal ID™ "Quantification" Results

Detection and Quantification of Ruminant-Associated Fecal Indicator Bacteria by Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Submitter: ABC Company
Date Received: October 3, 2011
Date Reported: October 11, 2011

SM #	Client #	Approximate Contribution of Ruminant Fecal Pollution in Water Sample	Comment
SM 16298	01012011E	**Major Contributor**	High levels of ruminant biomarker detected
SM 16300	01012011A	Negative	Negative for the ruminant biomarker

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Ruminant Fecal ID™ Quantification

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Submitter: ABC Company
Date Received: October 3, 2011
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SM #	Client #	Analysis Requested	General Marker Quantified*	Ruminant Specific Marker Quantified*	DNA Analytical Results
SM 16298	01012011E	Ruminant Fecal ID	5.44E+03	3.44E+02	**Positive**
SM 16300	01012011A	Ruminant Fecal ID			Negative

*Numbers reported as copy numbers per 100 mL of water

Laboratory Comments
Submitter: ABC Company
Report Date: October 11, 2011

Each submitted water sample was filtered for *Bacteroidetes* and the DNA was extracted and purified for DNA analysis. All reagents, chemicals and apparatus were verified and inspected beforehand to ensure that no false negatives or positives could be generated. In that regard, positive and negative controls were run to attest the integrity of the analysis. All inspections and controls tested negative for possible extraneous contaminates, including PCR inhibitors.

Samples 276 (Our Ref: SM 0226) and 278 (Our Ref: SM 0228) tested negative for the fecal *Bacteroidetes* ruminant gene biomarker. It is important to note that a negative result does not mean that the sample does not definitely have ruminant contamination. . The biomarkers serve as an indicator of the targeted fecal pollution, but the absence of the biomarker does not signify conclusively the absence of that form of fecal pollution. Only repeated sampling events (both during wet and dry events) will enable you to draw more definitive conclusions.

Samples 275 (Our Ref: SM 0225) and 277 (Our Ref: SM 0227) tested positive for the fecal *Bacteroidetes* ruminant gene biomarker suggesting that ruminant fecal contamination is present in these water samples. Nonetheless only repeated sampling events (both during wet and dry events) will enable you to draw more definitive conclusions.

DNA Analytical Method Explanation

Each submitted water sample was filtered through 0.45 micron membrane filters. Each filter was placed in a separate, sterile 5ml disposable tubes containing a unique mix of beads and lysis buffer and then bead beated for 5min. DNA extraction was prepared using the MoBio Power Water DNA Isolation kit (MoBio, Carlsbad, CA), as per manufacturer's protocol.

Amplifications to detect the target gene biomarker were run on an Applied Biosystems StepOne real-time thermal cycler (Applied Biosystems, Foster City, CA) in a final reaction volume of 20ul containing sample extract, forward primer, reverse primer, probe and an optimized buffer. All assays were run in duplicate. Absolute quantification was achieved by extrapolating target gene copy numbers from a standard curve generated from serial dilutions of known gene copy numbers.

For quality control purposes, a positive control consisting of ruminant fecal DNA and a negative control consisting of PCR-grade water, were run alongside the sample(s) to ensure a properly functioning reaction and to reveal any false negatives or false positives. The accumulation of PCR product was detected and graphed in an amplification plot. If the fecal indicator organism was absent in the sample, this accumulation was not detected and the sample was considered negative. If accumulation of PCR product was detected, the sample was considered positive..

Theory Explanation of Ruminant Fecal ID™ Quantification

The phylum *Bacteroidetes* is composed of three large groups of bacteria with the best-known category being *Bacteroidaceae* . This family of gram-negative bacteria is found primarily in the intestinal tracts and mucous membranes of warm-blooded animals and is sometimes considered pathogenic.

Comprising *Bacteroidaceae* are the genus *Bacteroides* and *Prevotella*. The latter genus was originally classified within the former (*i.e.Bacteroides*), but since the 1990's findings. *Bacteroides* and *Prevotella* are gram-negative, anaerobic, rod-shaped bacteria that inhabitant of the oral, respiratory, intestinal, and urogenital cavities of humans, animals and insects. They are sometimes pathogenic.

Fecal *Bacteroidetes* are considered for several reasons an interesting alternative to more traditional indicator organisms such as *E. coli* and *Enterococci*.[1] Since they are strict anaerobes, they are indicative of recent fecal contamination when found in water systems. This is a particularly strong reference point when trying to determine recent outbreaks in fecal pollution. They are also more abundant in feces of warm-blooded animals than *E. coli* and *Enterococci*. Furthermore, these latter two organisms are facultative anaerobes and as such they can be problematic for monitoring purposes since it has been shown that they are able to proliferate in soil, sand and sediments.

The Ruminant Fecal Quantification ID™ service is designed around the principle that fecal *Bacteroidetes* are found in large quantities in feces of warm-blooded animals.[2,3,4,5,6] Furthermore, certain categories of *Bacteroidetes* have been shown to be predominately detected in ruminants. Within these *Bacteroidetes*, certain genetic sequences in the *Bacteroides* and *Prevotella* genus have been found in ruminants.[7] As such, these bacterial strains can be used as indicators of ruminant fecal contamination.

One of the advantages of the Ruminant Fecal Quantification ID™ service is that the entire water is sampled and filtered for fecal *Bacteroidetes*. As such, this method avoids the randomness effect of culturing and selecting bacterial isolates off a petri dish. This is a particular advantage for highly contaminated water systems with potential multiple sources of fecal contamination.

Accuracy of the results is possible because the method uses PCR DNA technology. PCR allows quantities of DNA to be amplified into large number of small copies of DNA sequences. This is accomplished with small pieces of DNA called primers that are complementary and specific to the genomes to be detected. Through a heating process called thermal cycling, the double stranded DNA is denatured and inserted with complementary primers to create exact copies of the DNA fragment desired. This process is repeated rapidly many times ensuring an exponential progression in the number of copied DNA. If the primers are successful in finding a site on the DNA fragment that is specific to the genome to be studied, then billions of copies of the DNA fragment will be available and detected in real-time. Quantitative PCR (qPCR) adds a variant to the PCR process by inserting of a fluorescent probe within the primer set. This fluorescent probe serves as a molecular beacon for the quantification step. During each PCR cycle, quantitative PCR monitors the fluorescence emitted during the reaction. This is done in real-time during the first PCR cycles as a way to quantify the targeted gene. Absolute quantification is achieved by extrapolating target gene copy numbers from a standard curve generated from serial dilutions of plasmid DNA containing a known amount of the ruminant-specific biomarker. The Ruminant Fecal Quantification ID™ service uses qPCR to simultaneously confirm and quantify the ruminant- specific fecal *Bacteroidetes* genetic biomarker. This PCR technology avoids the cumbersome process of distinguishing DNA bands on a gel electrophoresis apparatus.

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